

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C12N 5/00, A61K 35/30, C12Q 1/00</b>		<b>A1</b>	(11) International Publication Number: <b>WO 96/15224</b>
			(43) International Publication Date: 23 May 1996 (23.05.96)
(21) International Application Number: <b>PCT/CA95/00636</b>		(74) Agent: <b>BENNETT JONES VERCHERE</b> , Suite 1800, 350 Albert Street, Box 25, Ottawa, Ontario K1R 1A4 (CA).	
(22) International Filing Date: 14 November 1995 (14.11.95)			
(30) Priority Data: 08/339,090 14 November 1994 (14.11.94) US 08/482,079 7 June 1995 (07.06.95) US		(81) Designated States: AL, AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, LS, MW, SD, SZ, UG).	
(60) Parent Applications or Grants (63) Related by Continuation US 08/339,090 (CIP) Filed on 7 June 1995 (07.06.95) US 08/482,079 (CIP) Filed on 14 November 1994 (14.11.94)		<b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(71) Applicant (for all designated States except US): <b>NEUROSPHERES HOLDINGS LTD.</b> [CA/CA]; 83 Heritage Medical Building, 3330 Hospital Drive, N.W., Calgary, Alberta T2N 4N1 (CA).			
(72) Inventors; and (75) Inventors/Applicants (for US only): <b>WEISS, Samuel</b> [CA/CA]; 4540 Chapel Road, N.W., Calgary, Alberta T2L 1A6 (CA). <b>REYNOLDS, Brent, A.</b> [CA/CA]; 235-11 A Street, N.W., Calgary, Alberta T2N 1X9 (CA).			
(54) Title: <b>IN VITRO INDUCTION OF DOPAMINERGIC CELLS</b>			
(57) Abstract <p>The expression of tyrosine hydroxylase in neural cells <i>in vitro</i> can be induced by contacting neural cells with a culture medium comprising at least one member of the fibroblast growth factor family in combination with conditioned medium or molecules of the transforming growth factor beta family. The method induces neural cells obtained from normally non-dopaminergic neural tissue such as the striatum and the cortex to express tyrosine hydroxylase. The cells can be used to treat neurological disorders in patients requiring dopaminergic cells.</p>			

ATTORNEY DOCKET NUMBER: 10177-211-999  
SERIAL NUMBER: 09/910,388  
REFERENCE: **B137**

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C12N 5/00, A61K 35/30, C12Q 1/00</b>		A1	(11) International Publication Number: <b>WO 96/15224</b>
			(43) International Publication Date: 23 May 1996 (23.05.96)
(21) International Application Number: <b>PCT/CA95/00636</b>			(74) Agent: <b>BENNETT JONES VERCHERE</b> , Suite 1800, 350 Albert Street, Box 25, Ottawa, Ontario K1R 1A4 (CA).
(22) International Filing Date: 14 November 1995 (14.11.95)			
(30) Priority Data: 08/339,090 14 November 1994 (14.11.94) US 08/482,079 7 June 1995 (07.06.95) US			(81) Designated States: AL, AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, LS, MW, SD, SZ, UG).
(60) Parent Applications or Grants (63) Related by Continuation US 08/339,090 (CIP) Filed on 7 June 1995 (07.06.95) US 08/482,079 (CIP) Filed on 14 November 1994 (14.11.94)			<b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(71) Applicant (for all designated States except US): <b>NEURO-SPHERES HOLDINGS LTD.</b> [CA/CA]; 83 Heritage Medical Building, 3330 Hospital Drive, N.W., Calgary, Alberta T2N 4N1 (CA).			
(72) Inventors; and (75) Inventors/Applicants (for US only): <b>WEISS, Samuel</b> [CA/CA]; 4540 Chapel Road, N.W., Calgary, Alberta T2L 1A6 (CA). <b>REYNOLDS, Brent, A.</b> [CA/CA]; 235-11 A Street, N.W., Calgary, Alberta T2N 1X9 (CA).			
(54) Title: <b>IN VITRO INDUCTION OF DOPAMINERGIC CELLS</b>			
(57) Abstract <p>The expression of tyrosine hydroxylase in neural cells <i>in vitro</i> can be induced by contacting neural cells with a culture medium comprising at least one member of the fibroblast growth factor family in combination with conditioned medium or molecules of the transforming growth factor beta family. The method induces neural cells obtained from normally non-dopaminergic neural tissue such as the striatum and the cortex to express tyrosine hydroxylase. The cells can be used to treat neurological disorders in patients requiring dopaminergic cells.</p>			

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/CA 95/00636

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9109936	11-07-91	US-A- 5411883	02-05-95
WO-A-9410292	11-05-94	AU-B- 5367694	24-05-94
		EP-A- 0669973	06-09-95
		FI-A- 952022	27-04-95
		NO-A- 951617	27-04-95
		WO-A- 9416718	04-08-94
WO-A-9301275	21-01-93	AU-B- 665012	14-12-95
		AU-B- 2242592	11-02-93
		CA-A- 2113118	21-01-93
		EP-A- 0594669	04-05-94
		FI-A- 935929	02-02-94
		JP-T- 6509225	20-10-94
		NO-A- 940056	03-03-94
		WO-A- 9416718	04-08-94
WO-A-9403199	17-02-94	AU-B- 4995193	03-03-94
WO-A-9306116	01-04-93	AU-B- 2681192	27-04-93
		CA-A- 2119463	01-04-93
		EP-A- 0610254	17-08-94
		FI-A- 941285	18-03-94
		HU-A- 66137	28-09-94
		JP-T- 7502980	30-03-95
		NO-A- 940922	19-05-94
		NZ-A- 244392	28-03-95
		PT-A- 100879	28-02-94
		ZA-A- 9207159	02-08-93

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LJ	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

### *In Vitro* Induction of Dopaminergic Cells

#### Priority Applications

This application is a continuation-in-part of U.S. Ser. No. 08/482,079, filed June 7, 1995, which is a continuation-in-part of U.S. Ser. No. 08/339,090, filed  
5 November 14, 1994.

#### Background of the Invention

It has been established that the phenotypic expression and survival of differentiating neural cells and the survival and metabolism of established neural cells is directed by a variety of extracellular signals. Neighboring cells and processes surrounding  
10 neural cells, play an important role in the regulation of cell differentiation, metabolic function and survival, largely through their release of growth and other regulatory factors.

Many neurological disorders, including Parkinson's disease, are the result of the degeneration of specific groups of neural cells. In the case of Parkinson's disease,  
15 the degeneration of a group of dopamine-containing cells, which connect the ventral tegmentum and substantia nigra (located in the ventral portion of the mesencephalon, or midbrain) with the striatum, have been implicated in the etiology of the condition.

In order to understand the factors which result in, or could prevent, the  
20 degeneration of these dopaminergic pathways, tissue obtained from the mesencephalon region has been extensively studied. Embryonic dopamine-containing neurons, derived from mesencephalic tissue, are difficult to culture as

the dopaminergic neurons do not survive well in culture. However, these cultures show an enhanced survival and/or a modified biochemical activity when cultured with a conditioned medium or when treated with growth factors. Embryonic tissue from the mesencephalon has been grown in conditioned culture media (CM) derived from the rat B49 glial cell line, the R 33 neural retina glial cell line and the JS Schwannoma cell line [Engel, J., *et al.*, *J. Neurosci. Res.*, 30: 359-371, (1991)]. In all three cases, CM significantly increased the survival of the cultured dopaminergic neurons. The enhanced survival of the dopaminergic neurons was not due to the proliferation of dopaminergic cells but was attributed to the effects of the CM on existing glial cells derived from the mesencephalic culture and the resultant interactions between the glial cells and the dopaminergic neurons, rather than to a direct effect on the dopaminergic neurons.

Culturing embryonic mesencephalic tissue in CM prepared from mesencephalic astrocytes, or co-culturing the tissue on a layer of mesencephalon-derived astrocytes, rescues dopaminergic neurons from death induced by serum deprivation. Astrocytes or the CM prepared from astrocytes from the striatum and cerebral cortex had significantly weaker protective effects [Takeshima *et al.* *J. Neurosci.*, 14(8): 4769-4779, (1994)]. In one report, CM derived from cortical astrocytes had no effect on dopaminergic cell survival or proliferation but did alter the biochemistry of this population of cells, resulting in a small increase in their uptake of dopamine [Gaul and Lübbert, *Proc. R. Soc. Lond. B*, 249: 57-63, (1992)].

Growth factors, many of which are present in CM from neural tissue, are believed to be responsible for regulating dopaminergic neuron survival and metabolism, either directly, or through their effects on adjacent cells. Growth factors reported to have a direct effect on dopaminergic neuron survival include interleukin-6 (IL-6) [Hama *et al.*, *Neurosci.*, 40(2): 445-452 (1991)], brain-derived neurotrophic factor (BDNF) [Hyman *et al.*, *Nature*, 350: 230-232, (1991)], basic fibroblast growth factor (FGF-2, formally referred to as bFGF) [Dal Toso *et al.* *J. Neurosci.*, 8(3): 733-745 (1988); Ferrari *et al.* *Dev. Biol.* 133: 140-147 (1989); and glial cell line-derived neurotrophic factor (GDNF), secreted by the rat B49 cell line [Lin, L.G.,

*et al.*, *Science*, 260: 1130-1132 (1993)], all of which specifically enhance the survival of dopaminergic neurons in dissociated rat or mouse embryo mesencephalon cultures without increasing neuron or glial cell numbers. GDNF dramatically increases the morphological differentiation of dopaminergic neurons, resulting in more extensive neurite outgrowth and increased cell body size. Some growth factors, such as nerve growth factor (NGF) (Hatanaka and Tsuki (1986), *Dev. Brains Res.* 30:47-56), platelet-derived growth factor (PDGF) and interleukin-1 (IL-1) (Engele & Bohn (1991), *Neurosci.* 11(10): 3070-3078); Mayer, E. (1993) *Dev. Brain Res.* 72: 253-258) and nerve growth factor (NGF) (Engele & Bohn, *ibid*) are reported to support dopaminergic cell survival in embryonic mesencephalic tissue through a glial cell-mediated mechanism.

*In vivo* studies indicate that the damage caused by mechanical or chemical lesions in the dopaminergic pathways between the mesencephalon and the striatum can be significantly reduced by treatment with epidermal growth factor (EGF) [Pezzoli *et al.*, *Movement Disorders* 6(4) 281-287, (1991)] and BDNF (Hyman *et al.*, *supra*). *In vitro* treatment with cyclic AMP, but not FGF-2 or NGF, increased the survival of cultured mesencephalic dopaminergic neurons in response to chemically induced degeneration produced by 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>) [Hyman *et al.*, *supra*; Hartikka *et al.*, *J. Neurosci. Res.*, 32: 190-201, (1992)]. Although the use of a FGF-2-stimulated astrocyte CM enhances dopamine uptake, the use of this CM did not have a protective effect when the neurons were chemically lesioned using MPP<sup>+</sup> (Gaul and Lübbert, *supra*).

Many of the cells obtained from embryonic tissue which normally gives rise to dopaminergic neurons (i.e. normally dopaminergic tissue), such as the mesencephalon and olfactory bulb, will eventually differentiate into dopaminergic neurons under primary culture conditions. However, an increased number of cells, in tissue obtained from normally dopaminergic areas of the brain, can be induced to differentiate into dopaminergic cells by co-culturing with feeder cell layers derived from neural tissue. The dopaminergic neurons of the olfactory bulb show a five-fold increase in number when embryonic olfactory bulb neurons are co-cultured with olfactory epithelial neurons. It is believed that a soluble factor,

calcitonin gene-related peptide (CGRP), which is present in the epithelial cells, is responsible for the induction of additional dopaminergic neurons in the olfactory bulb [Denis-Donini, *Nature*, 339: 701-703, (1989)]. Co-culturing rat embryonic neostriatal and substantia nigral tissue for one to three weeks on glial cell feeder  
5 layers obtained from the substantia nigra region induces the expression of dopaminergic cells as indicated by tyrosine hydroxylase immunoreactivity (TH+) in the tissue cultured from both areas. However, when the same tissues were co-cultured with glial cells from the neostriatum, dopaminergic cells were observed only in the substantia nigra tissue but not in the neostriatal tissue [Beyer *et al*,  
10 *Neurosci. Lett.*, 128: 1-3, (1991)]. The mechanism underlying the appearance of TH—IR in neostriatal tissue (an area which does not contain dopaminergic cells in the adult) was not determined. However, it could have been due to the induction of TH+ cells in the substantia nigral cell feeder layer in response to the presence of the striatal tissue; to the induction of dopaminergic properties in the striatal  
15 cells; or to promotion of the survival of dopaminergic cells in striatal tissue, such cells having been reported to occur transiently during development in the striatum (Tashiro *et al* (1989), *Neurosci. Lett.* 97: 6-10) and cortex (Sato and Suzuki (1990), *Dev. Brain Res.* 53: 1-5). Small numbers of TH+ cells (140 TH+ cells/cm<sup>2</sup>) have been induced in tissue from embryonic rat cortex using a  
20 combination of BDNF and dopamine, in a culture medium which contained 10% fetal calf serum. Fewer TH+ cells were seen when BDNF, or dopamine were used alone (Zhou *et al.* (1994), *Dev. Brain Res.* 81:318-324). A few cells from embryonic mouse striatal tissue can be induced to express TH+ when incubated with FGF-1 and an enhanced result can be obtained using a combination of FGF-1  
25 and an unidentified > 10 kD fraction obtained from muscle tissue (Du *et al.* (1994) *J. Neurosci.* 14(12): 7688-7694).

The degeneration of the substantia nigra dopaminergic neurons which characterizes Parkinson's Disease is normally treated using pharmacological interventions to augment the declining natural dopamine supply to the striatum. However, there  
30 are problems associated with drug treatment such as the development of tolerance to the medication and possible side effects. Neuronal grafts, using embryonic substantia nigral tissue have shown some potential for relieving experimentally



- induced Parkinsonism in rodents and primates and in some human Parkinsonian patients. However, graft survival is poor and only limited quantities of embryonic dopaminergic tissue are available. On average, 4-10 fresh, human embryos are required to obtain sufficient numbers of dopaminergic neurons for a single human
- 5 transplant (Widner *et al.*, N Engl J Med 327:1556-1563 (1992)). Preferred treatment would involve prevention of, or a reduction in the amount of the degeneration which occurs. Once damage has occurred, it would be preferable to replace the lost cells by implanting new dopaminergic neurons using cells derived from neural cells which have been proliferated in culture preferably from a non-
- 10 tumor cell line, or from cells that have not been intentionally immortalized in order to induce proliferation and, most preferably, would be derived from a patient's own neural tissue. Alternatively, a less invasive treatment would involve the *in vivo* manipulation of the patient's own population of neural cells in order to replace the function of the damaged dopaminergic neurons.
- 15 The prior art suggests that cultures of dopaminergic cells can be obtained through the use of glial feeder layers, or the application of certain growth factors or conditioned media to mesencephalic tissue and other dopaminergic tissues. These treatments can induce differentiation, increase the survival, or alter the metabolism of cells from normally dopaminergic tissue that has been cultured *in vitro*.
- 20 However, culture methods for inducing cells from other, non-dopaminergic brain regions to differentiate into dopaminergic cells are limited. While it has been demonstrated that a feeder layer of cells from regions such as the substantia nigra and olfactory bulb (areas which normally contain a relatively high population of dopaminergic cells) can be used to induce the appearance of dopaminergic cells in
- 25 certain embryonic central nervous system (CNS) tissues, there is no evidence that cells from non-dopaminergic neural tissue could be used as feeder layers in tissue culture designed to induce the appearance of dopamine in tissues, such as the striatum, which do not normally contain dopamine.

- For some purposes, especially transplantation and certain drug testing procedures,
- 30 it would be advantageous to use completely defined culture conditions to induce the differentiation of dopaminergic cells. It would be particularly advantageous if the

cells were obtained from both dopaminergic and normally non-dopaminergic neural tissue sources, thus maximizing the number of dopaminergic cells that can be generated from a single embryo.

There exists a need in the art for a reliable method of inducing neural cells,  
5 derived from all brain regions, from tissue obtained from animals of all ages, to differentiate into dopaminergic cells in the presence or absence of a feeder layer substrate. In particular, it would be advantageous to induce the expression of dopamine in cells derived from regions which do not normally contain dopaminergic cell bodies, such as the striatum, but which require dopamine for  
10 normal functioning.

Recently, it has been demonstrated that multipotent neural stem cells, obtained from embryonic and adult tissue, can be proliferated *in vitro* to generate large numbers of neural stem cell progeny, which, under appropriate conditions, can differentiate into neurons and glia (PCT applications No. WO 93/01275, WO  
15 94/16718, WO 94/10292, and WO 94/09119). It would be advantageous to generate dopaminergic cells from the proliferated progeny of multipotent neural stem cells, derived from any area of the CNS.

Accordingly, it is an object of this invention to provide a method for inducing large numbers of neural cells obtained from normally non-dopaminergic tissue to  
20 differentiate, *in vitro*, into dopaminergic cells in order to provide a reliable source of dopaminergic cells for various applications such as transplantation into patients with dopamine deficiencies and for drug screening procedures.

It is a further object of the invention to provide a method of inducing the undifferentiated, proliferated progeny of multipotent neural stem cells, derived  
25 from any area of the CNS known to contain such cells, to differentiate into dopaminergic cells, in order to provide unlimited quantities of dopaminergic cells for transplantation, drug screening and other purposes.

Additionally, it is an object of the invention to provide tissue culture methods that use completely defined culture conditions, and thus do not require the presence of a feeder layer of cells, conditioned medium, or serum, to increase the number of dopaminergic cells obtained from a single embryonic brain. Such cells would have  
5 use in specific applications such as transplantation into patients with dopamine deficiencies and for certain drug screening procedures.

These and other objects and features of the invention will be apparent to those skilled in the art from the following detailed description and the appended claims.

None of the foregoing references is believed to disclose the present invention as  
10 claimed and is not presumed to be prior art. The references are offered for the purpose of background information.

#### Summary of the Invention

- A method for inducing the expression of tyrosine hydroxylase in neural cells *in vitro* is disclosed. The method comprises contacting neural cells with a culture  
15 medium comprising at least one member of the fibroblast growth factor family and at least one additive selected from the group consisting of conditioned medium and molecules of the transforming growth factor beta family. The method induces neural cells obtained from normally non-dopaminergic neural tissue such as the striatum and the cortex to express tyrosine hydroxylase.
- 20 A method for treating a neurological disorder in a patient requiring dopaminergic cells is also disclosed. The method comprises contacting neural cells with a culture medium comprising at least one member of the fibroblast growth factor family and at least one member of the transforming growth factor beta family to produce differentiating or differentiated dopaminergic cells and transplanting the  
25 dopaminergic cells into the patient.

#### Description of the Drawings

**FIG. 1.** Dividing subependymal cells, in the subventricular zone of adult mice were labeled with repeated injections of BrdU over a 24 hour period. Within 30

minutes of the last injection, the mice were sacrificed and their brains removed. The subventricular zones were removed and the dissociated cells were cultured on poly-L-ornithine coated coverslips using complete medium with or without conditioned medium derived from the rat B49 glial cell line and FGF-2 (20 ng/ml).

- 5 Three days after plating, the cells were fixed and processed for dual-label indirect immunocytochemistry for TH (Eugene Tech, polyclonal 1:1000) and BrdU (Amersham, monoclonal, 1:100). BrdU-labelled cells (dividing subependymal cells) that were also TH-immunoreactive were seen only in the experiments in which the conditioned medium and growth factor were used. (A) A single BrdU-  
10 immunoreactive cell (arrow) that is TH-immunoreactive (B, arrow) suggesting that the adult proliferating subependymal cells can be induced to express TH in the presence of the conditioned medium and growth factor.

- FIG. 2.** Single, undissociated six day old primary generated neurospheres were plated onto poly-L-ornithine coated glass coverslips in DMEM/F12 hormone mix  
15 medium with conditioned medium derived from the rat B49 glial cell line and FGF-2 (20 ng/ml). Immunocytochemical analysis 24 hours later revealed the presence of TH+ cells. (A) Phase contrast micrograph of a neurosphere 24 hours post-plating. (B) The same sphere as in A, processed for TH-immunohistochemistry, reveals the presence of at least one TH+ cell, having  
20 neuronal morphology.

- FIG. 3.** Single, undissociated six day old second passage neurospheres were labelled with BrdU and plated onto a confluent bed of striatal-derived astrocytes. 24 hours post-plating, the cells were processed for dual-label indirect immunocytochemistry for TH (Eugene Tech, polyclonal 1:1000) and BrdU  
25 (Amersham, monoclonal, 1:100). (A) A BrdU-immunoreactive cell (arrow) is (B) TH-immunoreactive (arrow). (C) TH-immunoreactive cells (arrows) are also MAP-2 immunoreactive (D), demonstrating other neuronal characteristics, in addition to morphology.

Detailed Description of the Invention***In Vitro Induction of Dopaminergic Cells Derived from Primary Cells Obtained From Normally Non-Dopaminergic Neural Tissues***

- The term "dopaminergic neural tissue" refers to the tissue from regions of the CNS that are known, in the mature state, to contain significant numbers of dopaminergic cell bodies. Dopaminergic cells are neural cells whose presence in neural tissue is determined by the presence of tyrosine hydroxylase (TH) or the presence of dopamine decarboxylase and/or the absence of dopamine betahydroxylase within the cells, polymerase chain reaction techniques, or antibodies directed against dopamine. Tyrosine hydroxylase is the rate-limiting enzyme in the biochemical pathway which leads to dopamine production and is commonly used in the art as a marker for dopaminergic neurons. Dopaminergic neural tissue is found in regions of the retina, olfactory bulb, hypothalamus, dorsal motor nucleus, nucleus tractus solitarius, periaqueductal gray matter, ventral tegmenum, and substantia nigra.
- The term "normally non-dopaminergic neural tissue", as used herein, refers to the tissue from regions of the developed CNS that are not dopaminergic neural tissue.

- Using the methods disclosed herein, primary cells obtained from dissociated, neural tissue are induced to express tyrosine hydroxylase. The term "primary neural cell" refers to a cell obtained from neural tissue that has not been passaged *in vitro* (to a secondary culture). Primary neural cell cultures are prepared by removing tissue from an animal using any sterile procedure, dissociating the tissue to generate a suspension of primary cells, and placing the cells in any medium known to support the survival of cells. The primary cells are exposed to a culture medium comprising growth factors that induce dopamine production in cells obtained from normally non-dopaminergic neural tissue. The term "growth factor" refers to a biological factor (i.e. a biologically active substance functional in CNS cells) such as a protein, peptide, amino acid, lipid, carbohydrate, nucleic acid, nucleotide, or other substance having a growth, proliferative, differentiative, or trophic effect on neural cells, either singly, or in combination with other factors.
- A growth factor that induces dopamine production will bind to a receptor on a cell and induce the cell to begin to express, or increase its expression of messenger RNA (mRNA) for dopamine precursor molecules and enzymes involved in

dopamine production. A preferred growth factor is a member of the fibroblast growth factor family (e.g. FGF-1 or FGF-2), or equivalent growth factors that are capable of binding to FGF receptors on the cells. FGF and equivalent growth factors can be used singly or in combination with other growth factors. Growth factors are generally added at concentrations of about 1 to 100 ng/ml, usually about 5 ng/ml to 60 ng/ml. An optimum concentration is in the range of 10-30 ng/ml, with 20 ng/ml being most preferred. When a member of the FGF family is used, heparan sulphate, a glycosaminoglycan molecule which facilitates the binding of FGF to its receptors can be added to the culture medium at a concentration of 0.2  $\mu$ g/ml - 20  $\mu$ g/ml, preferably at a concentration of 0.4  $\mu$ g/ml - 4  $\mu$ g/ml. Most preferred is a concentration of about 2  $\mu$ g/ml. In a preferred embodiment, the culture medium comprises FGF in combination with a member of the transforming growth factor beta (TGF $\beta$ ) family. The TGF $\beta$  family includes basic myelin proteins (BMP-2, BMP-4, BMP-5, BMP-6, BMP-7), activins A & B, decapentaplegic (dpp), 60A, OP-2, dorsalin, GDFs (1, 3, and 9), nodal, MIS, Inhibin  $\alpha$ , transforming growth factors betas (TGF- $\beta$ 1, TGF- $\beta$ 2, TGF- $\beta$ 3, TGF- $\beta$ 5), and glial-derived neurotrophic factor (GDNF) (see Atrisano *et al.* (1994) J. Biochemica et Biophysica Acta vol. 1222:71-80).

If the TH+ cells are to be used for transplantation purposes or certain drug testing procedures, it is preferable to use a completely defined culture medium that has the nutrients and hormones necessary for supporting the survival of the cells. By "completely defined", it is meant that all of the components of the medium are known. Numerous defined culture media are commercially available. A preferred medium, referred to herein as "Complete Medium", comprises a 1:1 mixture of Dulbecco's Modified Eagle's Medium and F12 nutrient (GIBCO) plus 0.6% glucose, 2mM glutamine, 3mM sodium bicarbonate, 5mM HEPES buffer and a defined hormone mix and salt mixture (Sigma; 10% by volume) that includes 25 $\mu$ g/ml insulin, 100  $\mu$ g/ml transferrin, 20  $\mu$ M progesterone, 50  $\mu$ M putrescine, and 30nM selenium chloride.

The culture medium may comprise conditioned media (CM), which is a culture medium that has been exposed to living cells and thus contains substances, such as

growth factors, that have been released by the cells. However, the addition of CM renders the culture medium undefined, and thus is not preferred when the cells are to be used for transplantation purposes and certain drug testing procedures. CM can be obtained from any tissue that can be cultured that will induce dopamine  
5 expression in cells derived from normally non-dopaminergic neural tissue and/or will attenuate the effects of a dopamine-inducing growth factor. Any amount of CM can be used (0-100%). Generally, the culture medium will comprise from about 25 to 100% CM. Preferred CMs are derived from glial cells. Particularly preferred are CMs derived from the rat B49 glial cell line [Schubert *et al.*, *Nature*,  
10 249: 224, (1974)] and CM derived from astrocytes.

Primary cell cultures are plated, preferably at a density in the range of about  $10^2$  to  $10^7$  cells/ml, more preferably at a density of about  $10^6$  cells/ml. The cells can then be grown in any suitable container, such as a tissue culture flask, well, or petri dish. The container may or may not have a surface onto which cells can adhere.  
15 In cases where it is desirable that cells adhere, it is generally necessary to treat them with a substance that provides an ionically charged surface such as poly-D-lysine, poly-L-ornithine, Matrigel, laminin, fibronectin, and other surfaces known to induce cell attachment. The cells are capable of adhering to certain plastics. However, when glass substrates are used, it may be desirable to treat their  
20 surfaces. When adherence is not desired, glass substrates or some untreated plastic tissue culture substrates can be used. A poly-L-ornithine treated culture container provides a particularly suitable surface onto which the cells can adhere. Alternatively, the cells can be co-cultured on a feeder layer bed of any type or combination of types of cells as opposed to on a treated substrate. Preferred for  
25 co-culturing is a feeder bed of other neural cells such as neurons, astrocytes or oligodendrocytes derived from any region of the CNS.

The cultures are maintained as close to physiological conditions as possible. The pH should be between pH 6 to 8. Preferably between about pH 7.2 to 7.6, most preferably at a pH of about 7.4. Cells should be kept at a temperature close to  
30 physiological levels, between 30 to 40° C, more preferably between about 32 to 38° C and most preferably between about 35 to 37.5° C. The cells should be

maintained in about 5% CO<sub>2</sub>, 95% O<sub>2</sub>, and 100% humidity. However, culture conditions may be varied. For example, the addition of 1% fetal bovine serum (FBS) results in an increase in the number of TH+ neurons detected after cultures have been co-cultured on a glial cell feeder layer for 24 hours and also increases the numbers of TH+ cells detected when cells are grown in undefined culture medium in the absence of the feeder layer. One preferred embodiment is to grow primary cells in a culture medium containing CM derived from the rat B49 glial cell line and FGF-2 or a combination of EGF and FGF-2. A preferred embodiment for cells cultured in a defined medium for transplantation and other purposes is to grow primary tissue directly on a substrate-coated surface, such as poly-L-ornithine-coated coverslips, in a defined culture medium (e.g. Complete Medium) and a combination of FGF-2 with activin or BMP-2.

The induction of dopaminergic cells is determined using any method capable of measuring the presence of dopamine, such as immunocytochemistry using antibodies directed against dopamine, or measuring the biochemical activity of dopaminergic cells by measuring dopamine uptake. The presence of precursor molecules involved in the synthesis of dopamine can be measured. For example, immunocytochemical analysis to detect the presence of tyrosine hydroxylase, or assays, such as polymerase chain reaction and *in situ* hybridization techniques which detect mRNA for enzymes involved in dopamine synthesis are useful tools for measuring the presence of dopaminergic cells. The identification of dopaminergic neurons is accomplished using morphological analysis of neurons or by dual or triple labeling to show the presence of dopamine or dopamine precursors plus immunoreactivity for neuron specific enolase (NSE), the neurofilament proteins tau-1 and MAP-2 or for neu-N (a neuronal nuclear antigen) or  $\beta$ -tubulin and/or bromodeoxyuridine (BrdU), which labels actively dividing cells.



***In Vitro Induction of Dopaminergic Cells Derived from Multipotent Neural Stem Progeny Proliferated In Vitro From Embryonic and Adult Mammalian Neural Tissue***

- Multipotent neural stem cells have been reported and their potential use described
- 5 [Reynolds and Weiss, *Science*, 255: 1707 (1992); Reynolds *et al.*, *J. Neurosci.*, 12: 4565 (1992); Reynolds and Weiss, *Restorative Neurology and Neuroscience*, 4: 208 (1992); Reynolds and Weiss, *Neuronal Cell Death and Repair*, ed. Cuello (1993)]. Additionally, the utility of these cells is described in published PCT applications no. WO 93/01275, WO 94/16718, WO 94/10292, and WO 94/09119.
- 10 As used herein, the term "neural stem cell" refers to an undifferentiated multipotent neural stem cell that can be induced to proliferate *in vitro* in the presence of a proliferation-inducing growth factor such as, amphiregulin, acidic fibroblast growth factor (aFGF or FGF-1), basic fibroblast growth factor (bFGF or FGF-2), transforming growth factor alpha (TGF $\alpha$ ), and the like. The neural stem
- 15 cell is capable of self-maintenance, meaning that with each cell division, one daughter cell will also be a stem cell. The non-stem cell progeny (i.e. progenitor cells) of a single multipotent neural stem cell are capable of differentiating into neurons, astrocytes (type I and type II) and oligodendrocytes. Hence, the neural stem cell is "multipotent" because its progeny have multiple differentiative
- 20 pathways.

- The term "neural progenitor cell", as used herein, refers to an undifferentiated cell derived from a neural stem cell, that is not itself a stem cell. A distinguishing feature of a progenitor cell is that, unlike a stem cell, it has limited proliferative ability and thus does not exhibit self-maintenance. It is committed to a particular
- 25 path of differentiation and will, under appropriate conditions, eventually differentiate into glia or neurons.

The term "precursor cells", as used herein, refers to the progeny of neural stem cells, and thus includes both progenitor cells and daughter neural stem cells.

- CNS precursor cells derived from stem cells can be cultured using the methods
- 30 described in Example 3 below, and in the published PCT applications referenced

above. In the embryo, neural stem cell-containing tissue can be obtained from any CNS region, including the striatum, cortex, septum, thalamus, ventral mesencephalon and spinal cord. However, in adult, the neural tissue is preferably obtained from tissue lining the various ventricles and passageways within the CNS.

- 5 For example, tissue can be obtained from the regions immediately surrounding the lateral (first and second) ventricles and third ventricle of the forebrain, the cerebral aqueduct, the fourth ventricle, and the central canal. Growth factor-responsive stem cells from the neural tissue are grown in a culture medium in the presence of at least one growth factor. The medium is preferably a defined serum-free
- 10 medium. Growth factors which may be used for inducing proliferation, singly or in combination with other growth factors, include any growth factor which allows precursor cells to proliferate, including any molecule which binds to a receptor on the surface of the cell to exert a trophic, or growth-inducing effect on the cell. Such factors include acidic and basic fibroblast growth factors (FGF-1, and FGF-
- 15 2), epidermal growth factor (EGF), an EGF-like ligand, amphiregulin, transforming growth factor alpha (TGF $\alpha$ ), and the like. The cell is induced to divide giving rise to a cluster of undifferentiated cells that are not immunoreactive for the astrocyte marker, glial fibrillary acidic protein (GFAP); the neuronal markers, neurofilament (NF), microtubule associated protein (MAP-2), and neuron-specific enolase (NSE); or the oligodendrocyte markers, myelin basic protein
- 20 (MBP) and galactocerebroside (GalC). However, precursor cells within the cluster are immunoreactive for nestin, an intermediate filament protein found in undifferentiated CNS cells. The nestin marker was characterized by Lehndahl *et al.* [*Cell*, 60: 585-595 (1990)]. The mature phenotypes associated with the neural
- 25 cell types which may be differentiated from the progeny of the precursor cells are predominantly negative for the nestin phenotype.

- In the continued presence of a mitogen such as EGF, FGF or the like, precursor cells within the neurosphere continue to divide resulting in an increase in the size of the neurosphere and the number of undifferentiated cells [nestin(+), GFAP(-),
- 30 NF(-), MAP-2(-), NSE (-), MBP (-), GalC(-)]. At this stage, the cells are non-adherent and tend to form the free-floating clusters characteristic of neurospheres.

However, culture conditions may be varied so that while the precursor cells still express the nestin phenotype, they do not form the characteristic neurospheres.

Differentiation of the cells can be induced by any method known in the art which activates the cascade of biological events which lead to growth, which include the  
5 liberation of inositol triphosphate and intracellular  $\text{Ca}^{2+}$ , liberation of diacyl glycerol and the activation of protein kinase C and other cellular kinases, and the like. Treatment with phorbol esters, differentiation-inducing growth factors and other chemical signals can induce differentiation. Differentiation can also be induced by plating the cells on a fixed substrate such as flasks, plates, or coverslips  
10 coated with an ionically charged surface such as poly-L-lysine and poly-L-ornithine and the like.

Other substrates may be used to induce differentiation such as collagen, fibronectin, laminin, matrigel, and the like. Differentiation can also be induced by leaving the cells in suspension in the presence of a proliferation-inducing growth  
15 factor, without reinitiation of proliferation (i.e. without dissociating the neurospheres).

A preferred method for inducing differentiation of the neural stem cell progeny comprises culturing the cells on a fixed substrate in a culture medium that is free of the proliferation-inducing growth factor. After removal of the proliferation-  
20 inducing growth factor, the cells adhere to the substrate (e.g. poly-ornithine-treated plastic or glass), flatten, and begin to differentiate into neurons and glial cells. At this stage the culture medium may contain serum such as 0.5-1.0% fetal bovine serum (FBS). However, for certain uses, if defined conditions are required, serum would not be used. Within 2-3 days, most or all of the neural stem cell progeny  
25 begin to lose immunoreactivity for nestin and begin to express antigens specific for neurons, astrocytes or oligodendrocytes as indicated by immunoreactivity for MAP-2, GFAP and GalC, respectively, using immunocytochemistry techniques well known in the art.

In summary, CNS stem cells have been isolated from a variety of embryonic and adult CNS regions including the striatum, the spinal cord, brain stem, and hypothalamus. In each of these cases the isolated CNS stem cell exhibits self-maintenance and ultimately generates a large number of differentiated progeny which include neurons, astrocytes and oligodendrocytes. Thus, stem cells are present in multiple regions of the adult mammalian CNS and can be cultured, *in vitro* to obtain large numbers of undifferentiated neural cells, whose differentiation may be regulated by the application of growth factors and/or other biological factors. The undifferentiated cells (either as a cell suspension or intact neurospheres) proliferated using these techniques can be cultured to generate dopaminergic cells using the same methods described above for the induction of dopaminergic neurons in primary neural tissue.

#### *Transplantation of Cultured Dopaminergic Cells*

Therapeutic compositions comprising purified populations of differentiated dopaminergic cells, derived from primary culture or from the proliferated precursor progeny of neural stem cells can be prepared and administered into dopamine deficient regions of the brain of a recipient. Alternatively, therapeutic compositions comprising differentiating cells that have been cultured in a culture medium that induces the formation of dopaminergic cells may be prepared. The composition is administered to the appropriate brain region, where the cells are implanted prior to the completion of the differentiation process. Following implantation, the differentiation of dopaminergic cells may be completed *in vivo*. The composition may comprise purified cells, prepared using any suitable purification method. The composition may also comprise other types of neural cells. Any suitable method for the implantation of dopaminergic cells or precursor cells near the region of dopamine depletion may be used. Methods taught in U.S. Patent No. 5,082,670 to Gage *et al.* for the injection of cell suspensions, such as fibroblasts, into the CNS may be employed for the injection of the differentiated dopaminergic cells prepared by the culture methods disclosed herein. Additional approaches and methods may be found in *Neural Grafting in the Mammalian CNS*, Bjorklund and Stenevi, eds., (1987). Xeno and/or allografts may require the

application of immunosuppressive techniques or induction of host tolerance to enhance the survival of the implanted cells.

In some instances, it may be possible to prepare differentiating or differentiated dopaminergic cells from a recipient's own nervous system (for example, from  
5 tissue removed during biopsies). In such instances, the dopaminergic cells may be generated in culture from the progeny of neural stem cells. The cells from the dissociated nervous tissue are grown in the presence of a proliferation-inducing growth factor such as EGF or FGF. Upon suitable expansion of numbers, the precursor cells are contacted with a growth factor or a combination of growth  
10 factors and/or a conditioned medium or combinations of conditioned media, which induce the differentiation of dopaminergic cells. Preferably, the cells are proliferated and dopamine expression is induced using a defined culture medium. A composition comprising the differentiating or differentiated dopaminergic cells is administered to the appropriate region(s) of the recipient's brain. The composition  
15 may additionally comprise growth factors or other components that enhance the survival of the cells upon implantation.

#### *Drug Screening Using Cultured Dopaminergic Cells*

The dopaminergic cells produced using the methods disclosed herein may also be used to screen the effects of drugs and other compounds on dopaminergic cells.  
20 The screening methods disclosed in co-pending applications U.S. Ser. No. 08/311,099 and U.S. Ser. No. 08/339,090 may be used. Generally, the effect of the drugs and other compounds on the ability of the differentiating or differentiated cells to produce and/or metabolize dopamine would be measured.

#### Example 1: Propagation of Primary Cultures

25 The brains of E14 embryonic albino mice were placed in phosphate buffered saline (PBS) and dissected to obtain the striata, cerebral cortex and mesencephalon. The neural tissue was mechanically dissociated in serum-free medium (Dulbecco's Modified Eagle's Medium (DMEM) and F12 nutrient (GIBCO), using a fire polished Pasteur pipette. Cells were plated at a density of  $10^6$  cells/ml on poly-L-

ornithine-coated (15 $\mu$ g/ml; Sigma) glass coverslips in 24 well Nunclon culture dishes in a volume of 0.5 ml/well of Complete Medium or co-cultured on a glial feeder bed. Growth factors and/or conditioned media and/or 1% serum were added to the wells as outlined in Example 6. Cells were incubated at 37° C in a

5 95% air/5% CO<sub>2</sub> humidified atmosphere.

Example 2: Induction of Tyrosine Hydroxylase Expression  
in Cells Derived from the Adult Subventricular Zone

To label proliferating cells in the subependyma of the subventricular region of the brain, adult CD<sub>1</sub> mice were given five injections of BrdU (Sigma, 120 mg/kg) in

10 sterile saline solution, administered at two hour intervals [Morshead and van der Kooy, *J. Neurosci.*, 1: 49 (1992)]. Thirty minutes after the last BrdU injection, the animals were sacrificed. The striata were removed, cut into 1 mm coronal sections and placed in artificial cerebrospinal fluid (aCSF; 124 mM NaCl, 5 mM KCl, 1.3 mM MgCl<sub>2</sub>, 2mM CaCl<sub>2</sub>, 26 mM NaHCO<sub>3</sub> and 10 mM D-glucose (pH

15 7.35, ~280 mOsmol), aerated with 95% CO<sub>2</sub> at room temperature. After 15 minutes in the aCSF, the subventricular zones were micro dissected out, cut into small pieces and transferred to a spinner flask (Bellco Glass) with a magnetic stirrer, containing a low-Ca<sub>2</sub>-aCSF solution (124 mM NaCl, 5 mM KCl, 3.2 mM MgCl<sub>2</sub>, 0.1 mM CaCl<sub>2</sub>, 26 mM NaHCO<sub>3</sub> and 10 MM D-glucose (pH 7.35, ~280

20 mOsmol), 1.33 mg/ml of trypsin (9000 BAEF (benzoyl-L-arginine ethylester) units/mg), 0.67 mg/ml of hyaluronidase (2000 units/mg) and 0.2 mg/ml of kynurenic acid. This solution was aerated with 95% CO<sub>2</sub>/5% O<sub>2</sub> at 32°C to 35°C. After 90 minutes, tissue sections were transferred to normal aCSF for 5 minutes and then placed in DMEM/F12 medium containing 0.7 mg/ml ovomucoid (Sigma).

25 The tissue was mechanically triturated with a fire-narrowed Pasteur pipette. Cells were centrifuged at 400 r.p.m. for 5 minutes and resuspended in Complete Medium with or without conditioned medium from the rat B49 glial cell line (see example 5) and FGF-2 (20 ng/ml). They were plated onto poly-L-ornithine-coated glass coverslips in 24-well Nunclon tissue culture dishes and incubated at 37°C,

30 100% humidity, aerated with 95% CO<sub>2</sub>/5% O<sub>2</sub>, for three days. The cells were then fixed and processed for dual-label indirect immunocytochemistry for TH and BrdU, as outlined in Example 11. The presence of BrdU labeled cells, which were

also TH—IR, only in those cells treated with CM and FGF-2 (Fig. 1), suggest that adult proliferating subventricular zone cells can be induced to express TH in the presence of the CM and growth factor.

### Example 3: Isolation and Propagation of Embryonic Stem Cells

#### 5 A. Mouse Stem Cells

Embryonic day 14 (E14) CD<sub>1</sub> albino mice (Charles River) were decapitated and the brain and striata removed using sterile procedure. The tissue was mechanically dissociated with a fire-polished Pasteur pipette into Complete Medium. The cells were centrifuged at 800 r.p.m. for 5 minutes, the supernatant aspirated, and the  
10 cells resuspended in DMEM/F-12 medium for counting. The cells were resuspended in Complete Medium with 16-20 ng/ml EGF (purified from mouse submaxillary, Collaborative Research) or TGF $\alpha$  (human recombinant, Gibco), plated at  $0.2 \times 10^6$  cells/ml into 75 cm<sup>2</sup> tissue culture flasks (Corning) with no substrate pre-treatment and housed in an incubator at 37°C, 100% humidity, 95%  
15 air/5% CO<sub>2</sub>. The cells proliferated within the first 48 hours and by 3-4 days in vitro (DIV), they formed neurospheres that lifted off the substrate between 4-6 DIV.

After 7 DIV, the neurospheres were removed, centrifuged at 400 r.p.m. for 2-5 minutes, and the pellet was mechanically dissociated into individual cells with a  
20 fire-polished glass Pasteur pipet in 2 mls of Complete Medium.  $1 \times 10^6$  cells were replated into a 75 cm<sup>2</sup> tissue culture flask with 20 mls of the EGF-containing Complete Medium. The proliferation of the stem cells and formation of new neurospheres was reinitiated. This procedure can be repeated every 6-8 days.

#### B. Human Stem Cells

25 Fetal human forebrain tissue (post-conception week 10.5), obtained following routine suction abortion procedures, was mechanically dissociated with a fire-polished Pasteur pipette and placed into Complete Medium. The cells were centrifuged at 800 r.p.m. for 5 minutes, the supernatant aspirated, and the cells resuspended in DMEM/F-12 medium for counting. The cells were resuspended in  
30 Complete Medium with 20 ng/ml EGF (Chiron Corp.) and 10 ng/ml FGF-2 (R&D

Systems), plated at approximately  $1.5 \times 10^6$  cells/ml into culture flasks (Nunc T175) with no substrate pre-treatment and housed in an incubator at 37°C, 100% humidity, 95% air/5% CO<sub>2</sub>. The cells proliferated after 5 days and by day 10 started to form neurospheres which lifted off the substrate from day 21.

- 5 After 15 DIV, the neurospheres were removed and centrifuged at 1500 r.p.m. for 7 minutes. The pellet was mechanically dissociated into individual cells by triturating 150 times in 2 mls of Complete Medium. The cells were counted and  $1.5 \times 10^6$  cells/ml were replated into each of several culture flasks (Nunc T175), with 25 mls of Complete Medium containing EGF and FGF-2, as above.
- 10 The proliferation of the stem cells and formation of new neurospheres was reinitiated. This procedure can be repeated (i.e. the cells can be passaged) every 2-3 weeks.

- Example 4: Preparation of Glial Feeder Layers An astrocyte glial cell feeder layer was prepared from striatal tissue obtained from postnatal mice (0-24 hours). The
- 15 neural tissue was dissected out, minced and transferred to a 15 ml centrifuge tube containing DMEM/F12 (1:1)/10% FBS. The tissue was dissociated by trituration with a fire polished glass pipette and plated in Corning culture flasks containing 20 ml of DMEM/F12/10% FBS at a density of 150,000 cells/ml. When the primary astrocyte glial cells reached confluency, the cells were dissociated (using trypsin-
  - 20 EDTA) and replated at 200,000 cells/cm<sup>2</sup> onto poly-L-ornithine-coated glass coverslips in 24 well culture dishes. After 3 to 4 days, confluency was reestablished. Cells obtained from the culture of primary tissue (Example 1) or 6 day old, BrdU-labelled second passage neurospheres (Example 3) were washed twice and resuspended in fresh medium (free of serum, EGF or BrdU) prior to co-
  - 25 culturing the cells or neurospheres on the astrocyte feeder beds (Examples 6 and 7).

Example 5: Preparation of Conditioned Medium

- Conditioned medium (CM) was prepared from astrocytic cells or from a rat B49 glial cell line [Schubert *et al.*, *Nature*, 249: 224, (1974)]. An astrocyte glial cell
- 30 feeder layer was prepared (Example 4). The astrocytic layer was passaged once



before collection of the CM. Rat B49 glial cell line cells were cultured under the same conditions as the astrocytic cells. Confluent astrocyte or rat B49 glial cell line cultures were rinsed once with PBS and twice with serum-free Complete Medium and incubated in 20 ml of the same medium. The CM was collected 24, 5 48 and 72 hours after incubation commenced and centrifuged at 1,000 to 2,000 r.p.m. to remove any cell or debris. The CM was stored at -80°C.

Example 6: *In vitro* Induction of TH—IR in Cells Derived from Primary Culture

Paradigm 1: Primary cultures derived from tissue of the striatum and cerebral cortex were prepared as outlined in Example 1. Complete Medium (control), EGF 10 (20 ng/ml; Chiron), recombinant FGF-2 (20 ng/ml; R&D Systems), a combination of EGF plus FGF, astrocyte (ACM) or rat B49 glial cell line conditioned medium (BCM; see Example 5) were added singly or in combinations to individual wells at the time of plating (time, t=0). 1% FBS (Upstate Biotechnology Incorporated) was added to 50% of the wells which did not receive conditioned medium.

15 Immunocytochemical analysis for the detection of TH+ cells (Example 5) was undertaken 24 hours after plating. Results are summarized in Table 1. The addition of 1% FBS to the CM-free wells caused a three-fold increase in the numbers of TH+ cells recorded in the presence of the growth factors. The combination of FGF-2 plus BCM produced the most profound results, resulting in 20 the generation of, on average, more than approximately 5,000 TH+ cells/cm<sup>2</sup>.

TABLE I  
Average Number Of TH+ Cells Generated Per cm<sup>2</sup>  
From Striatal And Cortical Primary Cultures

5		STRIATUM		CORTEX	
		<u>FBS-free</u>	<u>1% FBS</u>	<u>FBS-free</u>	<u>1% FBS</u>
	control	4	2	12	29
	EGF	10*	18	14*	25*
	FGF	326	1128	72	295
	EGF+FGF	350	1668	75	229
10	ACM	10*	—	—	—
	ACM+EGF	10*	—	—	—
	ACM+FGF	1468	—	—	—
	ACM+E+F	1645	—	—	—
	BCM	16	—	—	—
15	BCM+EGF	26	—	—	—
	BCM+FGF	5397	—	—	—
	BCM+E+F	4470	—	—	—

\*Increase over control values not significant.

20 n = 4

Paradigm 2: Cells obtained from primary culture (Example 1) were washed twice and resuspended in fresh medium prior to co-culturing the cells on astrocyte feeder layers (Example 4) in the presence of Complete Medium (control) or Complete Medium plus EGF, FGF-2 or a combination of EGF and FGF-2 (20 ng/ml of each growth factor), 1  $\mu$ M BrdU and 1% FBS. Indirect immunocytochemistry (Example 11) was carried out on cells that had been cultured for 24 hours. A significant increase in TH-IR was detected in striatal cells co-cultured in the presence of FGF-2 or EGF plus FGF-2 when compared with cells cultured only in

Complete Medium. A small, but significant increase was observed in the presence of EGF alone. Primary cells derived from the cortex showed a similar response to EGF and FGF alone (a significant increase over control values) but the largest increase in TH—IR was seen using EGF and FGF-2 together. As indicated by

- 5 BrdU uptake, few cells from the mesencephalon are mitotically active, even in the presence of growth factors. The results are summarized in Table II.

TABLE II

Source of Primary Tissue	CONTROL Number of Cells/cm <sup>2</sup> TH + TH + BrdU +	EGF Number of Cells/cm <sup>2</sup> TH + TH + BrdU +	FGF Number of Cells/cm <sup>2</sup> TH + TH + BrdU +	EGF + FGF Number of Cells/cm <sup>2</sup> TH + TH + BrdU +
Cortex	50 1	143 3	148 25	216 46
Striatum	94 19	139 60	1475 460	1311 498
Mesencephalon	472 2	759 3	512 4	409 3

Example 7: In Vitro Induction of TH expression using a Defined Culture Medium

Primary cultures derived from tissue of the striatum and cerebral cortex were prepared as outlined in Example 1. Complete Medium (control), FGF-2 (20 ng/ml; R&D systems), BMP-2 (50ng/ml; Chiron Corp.) and activin (50ng/ml; Chiron Corp.) were added singly or in combination (Complete Medium plus FGF-2 and BMP-2 or Complete Medium plus FGF-2 and activin) to individual wells at the time of plating. Immunocytochemical analysis for the detection of TH+ cells (Example 11) was undertaken 24 hours after plating. Results are summarized in Table 3. The combination of FGF-2 plus activin or FGF-2 plus BMP-2 produced the most profound results, resulting in the generation of, on average, approximately 5,000 TH+ neurons per cm<sup>2</sup>. In contrast, the control, comprising Complete Medium alone, produced an average of 2 TH+ cells per cm<sup>2</sup>.

Table 3: Number of TH+ immunoreactive per cm<sup>2</sup>

MEDIUM SUPPLEMENT	#TH+CELLS
No supplement (control)	2±1
FGF-2	210±29
BMP <sub>2</sub>	2±1
Activin	0
FGF-2 + BMP <sub>2</sub>	4622±1207
FGF-2 + Activin	4930±289

Example 8: In Vitro Induction of TH—IR in Neural Stem Cell-Derived Precursor Cell Progeny Using Conditioned Medium

Paradigm 1: Single, undissociated 6 day old primary generated neurospheres (see Example 3) were plated onto poly-L-ornithine coated glass coverslips, in Complete Medium with or without rat B49 glial cell line-derived CM (Example

5) + 20 ng/ml FGF-2, and incubated at 37° C in 5% CO<sub>2</sub>, 95% air, 100% humidity. 24 hours after plating, indirect immunocytochemistry for TH+ (see Example 11) revealed the presence of TH+ cells, with processes and a neuronal morphology, in the wells containing CM and FGF-2 (FIG. 2) but not in the wells containing only Complete Medium.

Paradigm 2: Single, undissociated six day old, second passage neurospheres (Example 3) were labeled with BrdU, washed twice and resuspended in fresh medium prior to co-culturing the neurospheres on astrocyte feeder layers (Example 4) in the presence of Complete Medium or Complete Medium plus FGF-2 (20 ng/ml). Indirect immunocytochemistry (Example 11) was carried out on cells that had been cultured for 24 hours. TH—IR cells with neuronal morphology that stained positive for MAP-2 were observed in the neurospheres cultured with FGF-2. Several of the TH+ cells showed BrdU immunoreactivity (FIG. 3).

15 Example 9: *In Vitro* Induction of TH—IR in Neural Stem Cell-Derived Progeny Using Defined Medium

Single, undissociated 6 day old primary generated neurospheres (see Example 3) are plated onto poly-L-ornithine coated glass coverslips, in Complete Medium with a combination of 20 ng/ml FGF-2 and 20 ng/ml BMP-2 or a combination of 20 ng/ml FGF-2 and 20 ng/ml activin (Example 7), and incubated at 37° C in 5% CO<sub>2</sub>, 95% air, 100% humidity. The number of dopaminergic neurons is determined by TH-immunoreactivity (Example 11).

Example 10: *In Vitro* Induction of TH—IR in Human Embryonic Neural Stem Cell-Derived Progeny

25 Human neural stem cells were proliferated and passaged 35 times, to increase the quantity of cells, as described in Example 3B. 12 day old neurospheres were obtained from the final passage. The neurospheres were washed, suspended and mechanically dissociated in Complete Medium. The neurospheres were plated on poly-L-ornithine coated (15 µg/ml) glass coverslips (density: 0.3 million cells) in 24 well Nunclon culture dishes under either control (Complete Medium) or TH-inducing conditions (0.8 ml/well; 75% rat B49 glial cell line-derived CM (Example 5) + 20 ng/ml FGF-2). In addition, cell preparations containing CM

alone (75%) or FGF-2 (20 ng/ml) alone were incubated to determine their effects when used separately. The cells were incubated at 37°C in 5% CO<sub>2</sub>, 95% air, 100% humidity. After 1 DIV and 3 DIV the number of TH-IR cells were determined as outlined in Example 11. The results are shown in Table 4.

5

Table 4: Number of TH+ Cells/cm<sup>2</sup>

10

CONDITIONS	Number of TH <sup>+</sup> Cells/cm <sup>2</sup> After 1 Day <i>In Vitro</i>	Number of TH <sup>+</sup> Cells/cm <sup>2</sup> After 3 Days <i>In Vitro</i>
Complete Medium (control)	278	278
FGF-2 at 20 ng/ml	265	222
CM (75%)	667	667
CM (75%) + FGF-2 (20 ng/ml)	473	1890

Example 11: Immunocytochemistry

- 15 Cells were fixed with 4% paraformaldehyde for 30 minutes followed by three washes in PBS for 10 minutes each. The cells were incubated with a primary anti TH antibody (rabbit polyclonal, 1:1,000, Engene Tech International Inc.; or 1:100, Pel-freeze) prepared in PBS/10% normal goat serum/0.3 Triton X-100 for 2 hours at 37°C. Following three rinses in PBS, goat anti-rabbit rhodamine (Jackson) was
- 20 applied in PBS for 30 minutes at room temperature. In some cases, MAP-2 (Boehringer-Mannheim) was used to identify neurons. Cells were then washed three times for 10 minutes each in PBS, rinsed with water, placed on glass slides and coverslipped using Fluorosave (Calbiochem) as the counting medium. The number of dopaminergic neurons was determined by counting all the TH-
- 25 immunoreactive (TH+) per cm<sup>2</sup>, under 200x magnification.

Example 12: Transplantation of Dopaminergic CellsA: Transplantation of Cells Derived from Cultured Human Neural Stem Cells into a Mouse Model of Parkinson's Disease

- Fetal human forebrain stem cells are proliferated in culture as outlined in
- 5 Example 3B and passaged 35 times. Three days prior to transplantation, the floating neurospheres are removed and treated in one of two methods to enhance neuronal differentiation to a TH phenotype. With the first TH enhancement method, the cells are treated as described in Example 10 above (with 1  $\mu$ M BrdU added to the medium). On the day of transplantation, cells are rinsed, detached
- 10 with trypsin/EDTA, and then treated with trypsin inhibitor. Cells are suspended in HBSS at a density of  $20 \times 10^6$  cells/ml for transplantation. For the second TH enhancement method, the cells are placed in untreated flasks, which causes neurospheres to remain floating. The culture medium components are the same as in the first method. On the day of transplantation, cells are rinsed, triturated
- 15 lightly, and then suspended in HBSS at a density of  $21 \times 10^6$  cells/ml for transplantation. All cells are stored at 4 °C during the transplantation period.

- To model Parkinson's disease, host Wistar rats (approx. 275 gm; Charles River) receive unilateral administration of 4  $\mu$ l of a 2  $\mu$ g/ $\mu$ l solution of 6-OHDA (6-hydroxydopamine; Sigma) to lesion the dopaminergic neurons in the substantia
- 20 nigra compacta of the ipsilateral side. Sixteen days later they receive transplants of the human stem cell progeny into the ipsilateral striatum. 5 animals receive cells treated as in the first TH enhancement method, and 5 animals receive cells treated as in the second enhancement method. Animals are sacrificed after 1 week, 6 weeks, and 3 months.

- 25 Animals are perfused transcardially with aldehydes, brain tissue is removed and cut into 10  $\mu$ m thick sections and then tissue is mounted directly onto microscope slides. Double immunostaining techniques are used for light microscopy to identify transplanted cells (BrdU+) that contain tyrosine hydroxylase (TH+) using antibodies against BrdU and TH. Different colored substrates allow the
- 30 identification of double labeled cells indicating that a population of the transplanted cells differentiated into neurons with a dopaminergic phenotype.



B: Parkinson's disease is a condition characterized by degeneration of the dopaminergic pathway to the striatum and results from decreased dopamine levels in this region. The condition is characterized by muscular rigidity, tremor, and other motor abnormalities. Precursor cells, prepared from human fetal tissue are proliferated (Example 3B) and induced to differentiate into dopaminergic cells (Example 11). Dopaminergic cells are stereotactically injected into the striatum of a Parkinsonian patient. Booster injections may be performed if required. Alternatively, precursor cells are prepared from neural tissue obtained from a biopsy of the Parkinsonian patient's brain and induced to differentiate into dopaminergic cells (Example 7 or 8) and stereotactically injected into the striatum of the patient. Alternatively, the precursor cells can be cultured in the presence of a culture medium that induces the formation of dopaminergic cells and implanted into the striatum of a Parkinsonian patient where the precursor cells differentiate, *in vivo*, into dopaminergic cells. Improved motor control is used to measure the success of the transplantation.

Example 12: Drug Screening Using Cultured Dopaminergic Cells

Prozac, a drug widely used in the treatment of psychiatric illness, is added to cultured dopaminergic cells prepared as outlined in Examples 6, 7, 8, 9, 10 in concentrations ranging from 1 ng/ml to 1000 ng/ml. The effects of the drug at various concentrations with respect to cell metabolism and survival is monitored.

All references, patents, and patent applications cited herein are incorporated herein by reference.

## WHAT IS CLAIMED IS:

1. A method for inducing the expression of tyrosine hydroxylase in neural cells *in vitro* comprising contacting said neural cells with a culture medium comprising at least one member of the fibroblast growth factor family and at least one additive  
5 selected from the group consisting of conditioned medium and members of the transforming growth factor beta family.
2. The method of claim 1 wherein said culture medium is defined, said member of the fibroblast growth factor family is FGF-2 and said additive is a member of the transforming growth factor beta family selected from the group consisting of  
10 activin and bone morphogenic protein-2.
3. The method of claim 1 wherein said neural cells are cultured on an ionically charged surface selected from the group consisting of poly-D-lysine, poly-L-ornithine, Matrigel, laminin, fibronectin.
4. The method of claim 3 wherein said charged surface is poly-L-ornithine.
- 15 5. The method of claim 1 wherein said neural cells are obtained from normally non-dopaminergic neural tissue.
6. The method of claim 5 wherein said normally non-dopaminergic neural tissue is selected from the group consisting of the striatum and the cortex.
7. The method of claim 1 wherein said additive is conditioned medium selected  
20 from the group consisting of astrocyte conditioned medium and glial cell conditioned medium.
8. The method of claim 7 wherein said conditioned medium is derived from rat B49 glial cell line.
9. The method of claim 1 wherein said culture medium further comprises serum.

10. The method of claim 1 wherein said neural cells are cultured in the absence of a feeder layer.
11. The method of claim 1 wherein said neural cells are primary cells obtained from embryonic tissue.
- 5 12. The method of claim 1 wherein said neural cells are the progeny of at least one multipotent neural stem cell proliferated *in vitro* in the presence of a proliferation-inducing growth factor.
13. The method of claim 12 wherein said multipotent neural stem cell is derived from adult neural tissue.
- 10 14. A method for inducing the expression of tyrosine hydroxylase in primary neural cells *in vitro* comprising contacting said neural cells with an ionically charged surface selected from the group consisting of poly-D-lysine, poly-L-ornithine, Matrigel, laminin, fibronectin, in a defined culture medium comprising a member of the fibroblast growth factor family and at least one member of the
- 15 transforming growth factor beta family.
15. The method of claim 14 wherein said member of the fibroblast growth factor family is FGF-2 and said member of the transforming growth factor beta family is selected from the group consisting of activin and bone morphogenic protein-2.
16. The method of claim 14 wherein said primary neural cells are obtained from
- 20 normally non-dopaminergic neural tissue.
17. A method for inducing the expression of tyrosine hydroxylase in neural cells obtained from normally non-dopaminergic neural tissue comprising contacting said neural cells with a feeder layer and a culture medium comprising fibroblast growth factor.

18. The method of claim 17 wherein said feeder layer is derived from normally non-dopaminergic neural tissue.
19. The method of claim 17 wherein said culture medium further comprises serum.
- 5 20. The method of claim 17 wherein said neural cells are the progeny of at least one multipotent neural stem cell proliferated *in vitro* in the presence of a growth factor.
21. The method of claim 17 wherein said normally non-dopaminergic tissue is selected from the group consisting of the striatum and the cortex.
- 10 22. The method of claim 17 wherein said culture medium further comprises conditioned medium.
23. The method of claim 22 wherein said conditioned medium is derived from glial cells.
24. The method of claim 22 wherein said conditioned medium is derived from  
15 astrocytes.
25. The method of claim 22 wherein said conditioned medium is derived from rat B49 glial cell line.
26. The method of claim 18 wherein said culture medium further comprises serum.
- 20 27. A method of treating a neurological disorder in a patient requiring dopaminergic cells comprising transplanting differentiating or differentiated dopaminergic cells into said patient at a CNS region requiring dopaminergic cells, said differentiating or differentiated dopaminergic cells being formed by contacting neural cells with a culture medium comprising a member of the fibroblast growth

factor family and at least one member of the transforming growth factor beta family.

28. The method of claim 27 wherein said patient has Parkinson's disease and said differentiating or differentiated dopaminergic cells are administered to said patient's  
5 striatum.

29. The method of claim 27 wherein said member of the fibroblast growth factor family is FGF-2 and said member of the transforming growth factor beta family is selected from the group consisting of activin and bone morphogenic protein.

30. The method of claim 27 wherein said culture medium is defined.

10 31. The method of claim 27 wherein said neural cells are obtained from the CNS of said patient.

32. A method of screening the effects of a drug on dopaminergic cells comprising  
a) culturing neural cells derived from neural tissue in a culture medium comprising at least one member of the fibroblast growth factor family and at least  
15 one additive selected from the group consisting of conditioned medium, and members of the transforming growth factor beta family to produce dopaminergic cells,

b) administering a drug to said dopaminergic cells, and

c) observing the effects of said drug on said dopaminergic cells.

20 33. The method of claim 32 wherein said effects are determined by measuring the ability of said dopaminergic cells to produce or metabolize dopamine.

34. A therapeutic composition for the treatment of a neurological disease or disorder, said composition comprising differentiating or differentiated dopaminergic cells derived from neural cells cultured *in vitro* in a culture medium comprising a  
25 member of the fibroblast growth factor family and at least one member of the transforming growth factor beta family.

35. The therapeutic composition of claim 34 wherein said member of the fibroblast growth factor family is FGF-2 and said member of the transforming growth factor beta family is selected from the group consisting of activin and bone morphogenic protein.
- 5 36. The therapeutic composition of claim 34 or 35 wherein said culture medium is defined.
37. The therapeutic composition of any of claims 34 to 26 wherein said neural cells are obtained from the CNS of said patient.



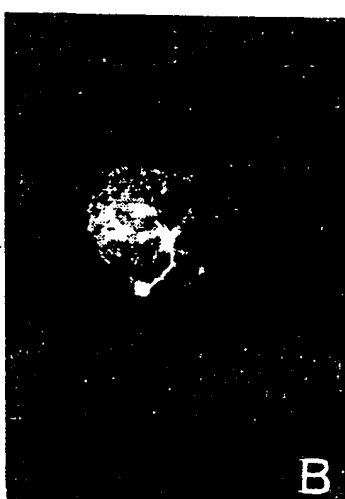
**FIG. 1A**



**FIG. 1B**



**FIG. 2A**



**FIG. 2B**

FIG. 3B

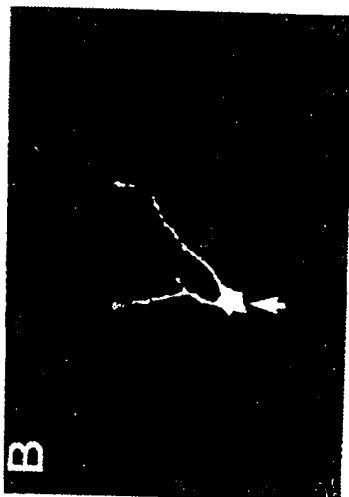


FIG. 3D



FIG. 3A

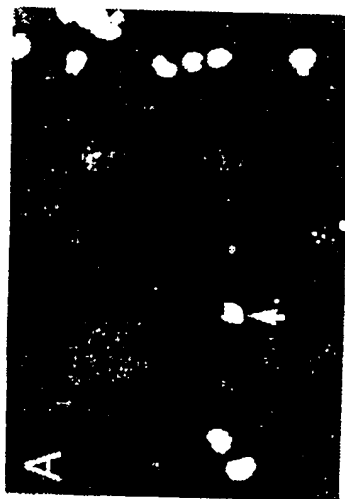
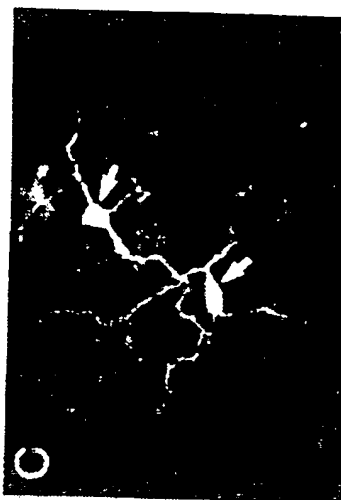


FIG. 3C





# INTERNATIONAL SEARCH REPORT

International Application No  
PC1/CA 95/00636

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC 6 C12N5/00 A61K35/30 C12Q1/00		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) IPC 6 C12N A61K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EXPERIMENTAL NEUROLOGY, vol. 128, 1994 NEW YORK, pages 59-76, SILANI V. ET AL 'Development of dopaminergic neurons in the human mesencephalon and in vitro effects of basic fibroblast growth factor treatment' see the whole document	1,3-7, 9-13, 17-24, 26,32,33
Y	---	8,25
	-/--	
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search  28 February 1996		Date of mailing of the international search report  25. 03. 96
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016		Authorized officer  Fernandez y Branas, F

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/CA 95/00636

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF NEUROSCIENCE RESEARCH, vol. 30, 1991 NEW YORK, pages 359-371, ENGELE, J. ET AL 'Conditioned media derived from glial cell lines promote survival and differentiation of dopaminergic neurons in vitro: role of mesencephalic glia' see the whole document ---	8,25
X	THE NEW ENGLAND JOURNAL OF MEDICINE, vol. 327, no. 22, 1992 BOSTON, pages 1556-1563, WIDNER H. ET AL 'Bilateral fetal mesencephalic mgrafting in two patients with parkinsonism induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridi ne (MPTP)' cited in the application see the whole document ---	27-31, 34-37
X	WO,A,91 09936 (HANA BIOLOGICS INC.) 1991  see page 4 - page 5 ---	27-31, 34-37
X,P	THE JOURNAL OF NEUROSCIENCE, vol. 14, no. 12, December 1994 BALTIMORE, pages 7688-7694, XINYU DU ET AL 'Novel expression of the tyrosine hydroxylase gene requires both acidic fibroblast growth factor and an activator' see the whole document ---	1,5,6,11
A	WO,A,94 10292 (NEUROSPHERES LTD) 11 May 1994 see the whole document ---	1-37
A	WO,A,93 01275 (WEISS S) 1993 see the whole document ---	1-37
A	WO,A,94 03199 (REGENERON PHARMACEUTICALS INC) 17 February 1994 see the whole document ---	1-37
A	WO,A,93 06116 (SYNTEX-SYNERGEN NEUROSCIENCE JOINT VENTURE) 1993 see the whole document ---	1-37
A	NATURE, vol. 344, 1990 LONDON GB, pages 868-870, SCHUBERT D. ET AL 'Activin is a nerve cell survival molecule' see the whole document -----	1-37

1

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA95/00636

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 27-31  
because they relate to subject matter not required to be searched by this Authority, namely:  
REMARK: ALTHOUGH CLAIMS 27-31 ARE DIRECTED TO A METHOD OF TREATMENT OF THE HUMAN/ANIMAL BODY THE SEARCH HAS BEEN CARRIED OUT AND BASED ON THE ALLEGED EFFECTS OF COMPOSITION.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.